APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1993, p. 2918–2926
0099-2240/93/092918-09$02.00/0

Vol. 59, No. 9

Pyrobaculum aerophilum sp. nov., a Novel Nitrate-Reducing Hyperthermophilic Archaeum

PAUL VÖLKL,[1] ROBERT HUBER,[1] ELISABETH DROBNER,[1] REINHARD RACHEL,[1] SIEGFRIED BURGGRAF,[1] ANTONIO TRINCONE,[2] AND KARL O. STETTER[1]*

Archaeenzentrum, Universität Regensburg, 93053 Regensburg, Germany,[1] and Istituto per la Chimica di Molecole di Interesse Biologico, Arco Felice, Naples, Italy[2]

Received 16 February 1993/Accepted 8 July 1993

A novel rod-shaped hyperthermophilic archaeum has been isolated from a boiling marine water hole at Maronti Beach, Ischia, Italy. It grew optimally at 100°C and pH 7.0 by aerobic respiration as well as by dissimilatory nitrate reduction, forming dinitrogen as a final product. Organic and inorganic compounds served as substrates during aerobic and anaerobic respiration. Growth was inhibited by elemental sulfur. The cell wall was composed of a surface layer of hexameric protein complexes arranged on a p6 lattice. The core lipids consisted mainly of glycerol diphytanyl glycerol tetraethers with various degrees of cyclization. The G+C content was 52 mol%. The new isolate resembled members of the genera *Thermoproteus* and *Pyrobaculum* by its ability to form characteristic terminal spherical bodies ("golf clubs"). On the basis of its 16S rRNA sequence, the new isolate exhibited a close relationship to the genus *Pyrobaculum*. It is described as a new species, which we name *Pyrobaculum aerophilum* (type strain: IM2; DSM 7523).

The respiratory metabolism of hyperthermophilic bacteria and archaea (36) presents an interesting set of questions related to their phylogeny. Among the bacteria, aerobic respiration is common. Even the organism with the highest growth temperature of this domain, *Aquifex pyrophilus*, is a facultative aerobe (16). It represents the deepest branching lineage within the bacterial domain (8) and grows obligately autotrophically by the Knallgas reaction with hydrogen and oxygen. Alternatively, it is able to grow anaerobically by dissimilatory nitrate reduction, a mode of anaerobic respiration that is widespread among the mesophilic facultatively anaerobic bacteria (40).

The domain *Archaea* contains the organisms with the highest growth temperatures, and within this domain, aerobic respiration is rare (28, 36). The physiologically and metabolically diverse *Euryarchaeota* kingdom comprises hyperthermophiles, thermophiles, and mesophiles. Within this kingdom, the only aerobic organisms are represented by members of the thermoacidophilic genus *Thermoplasma* (9, 24) and the mesophilic extreme halophiles (29). They are "fast-clock" organisms of long evolutionary lineages (34). In addition to aerobic respiration, some extreme halophiles (e.g., *Halobacterium denitrificans*) are able to grow anaerobically by dissimilatory nitrate reduction with organic substrates (30, 40).

The *Crenarchaeota* kingdom represents the sulfur-metabolizing phylogenetic branch of the *Archaea* with the two orders *Sulfolobales* (25, 26) and *Thermoproteales* (15, 38). It consists exclusively of extreme thermophiles and hyperthermophiles with growth temperatures of up to 110°C (27). The thermoacidophilic *Sulfolobales* represent the longest evolutionary lineage within the *Crenarchaeota*. The cells are irregularly shaped cocci and grow aerobically by sulfur oxidation and strictly anaerobically by sulfur reduction, depending on the genus (25). Recently, chemolithoautotrophic growth with hydrogen and oxygen (Knallgas reaction) was found for the first time within the *Archaea* in the *Sulfolobales* (13). All members of the *Thermoproteales* are strict anaerobes. The rod-shaped representatives of this order belong to the three genera *Thermofilum* (37), *Thermoproteus* (38), and *Pyrobaculum* (14). So far, they have been exclusively isolated from low-salt continental hydrothermal areas. They grow either chemolithoautotrophically by sulfur reduction or organotrophically by sulfur respiration or by fermentation (6, 14, 15). For all members of the *Crenarchaeota*, dissimilatory nitrate reduction is so far unknown.

Here, we report the isolation and characterization of a novel hyperthermophilic, facultatively anaerobic, denitrifying archaeum, which is the first aerobic representative within the *Thermoproteales*.

MATERIALS AND METHODS

Strains and culture conditions. *Thermoproteus tenax* DSM 2078, *Thermoproteus neutrophilus* DSM 2338, *Pyrobaculum islandicum* DSM 4184, and *Pyrobaculum organotrophum* DSM 4185 were from the culture collection of our institute. They were grown as described previously (12, 14, 38). If not mentioned otherwise, isolate IM2 was cultivated in BS medium, containing the following (per liter of double-distilled H_2O): $NaHCO_3$, 2.2 g; NH_4Cl, 0.25 g; KH_2PO_4, 0.07 g; $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$, 2 mg; $(NH_4)_2Ni(SO_4)_2 \cdot 2H_2O$, 2 mg; $NaSeO_4$, 0.1 mg; $Na_2WO_4 \cdot 2H_2O$, 0.1 mg; trace mineral solution (1), 10 ml; marine medium (KNO_3 omitted [39]), 125 ml; synthetic sea water (18), 125 ml. For determination of salt dependence of growth, the salt concentration in the medium was adjusted with NaCl. pH was adjusted to 7.0 with H_2SO_4. BSY medium contained 0.05% yeast extract (Bacto; Difco, Detroit, Mich.) in addition. The final concentration of other substrates used was 0.05% (wt/vol). If not mentioned otherwise, the KNO_3 and KNO_2 concentrations were 0.1 and 0.01% (wt/vol), respectively. Strictly anaerobic culture medium was obtained according to the anaerobic technique of Balch and Wolfe (2). Oxygen was reduced by adding 0.05% $Na_2S \cdot 9H_2O$ with resazurin (5 μg/l) as the redox indicator. Microaerobic medium was prepared as described previously (16). Prior to autoclaving, the medium

* Corresponding author.

was dispensed in 10-ml aliquots into 120-ml serum bottles which were stoppered, and the gas phase was exchanged with the desired gas mixture. As gas phases, H_2-CO_2 or N_2-CO_2 (300 kPa; 80:20, vol/vol) was used routinely. For aerobic cultivation with organic and inorganic substrates, 1 or 0.6% O_2 (by volume) was added to these gas phases.

Metabolic studies and analyses of end products were performed in a 1-liter glass fermentor containing 700 ml of medium. The fermentor was operated at 97°C and stirred with 150 rpm without overpressure. For aerobic growth conditions, it was aerated with filter-sterilized air (20 ml/min). For anaerobic growth, the medium was gassed with approximately 30 ml of N_2-CO_2 (80:20, vol/vol) per min.

For plating, BSY medium was solidified with 0.6% Gelrite (Kelco, San Diego, Calif.). The plates were incubated in a pressure cylinder (1) under N_2-CO_2 (80:20; 200 kPa) for anaerobic growth and under approximately 100 kPa of air (atmospheric pressure) plus 100 kPa of N_2-CO_2 (80:20) for aerobic growth.

Cell masses were grown anaerobically at 100°C under stirring (150 rpm) in a 300-liter enamel-protected fermentor (HTE Bioengineering, Wald, Switzerland) pressurized with 100 kPa of N_2-CO_2 (80:20, vol/vol) and continuous gassing (2.5 liters of N_2-CO_2 per min). Packed cell masses exhibited a dark-green color. Under aerobic growth conditions, the fermentor was operated at 97°C without overpressure by aerating (2.5 liters of air per min) and stirring (150 rpm). Cell masses showed a light brown color with a touch of green.

Light and electron microscopy. Light microscopy and photography were carried out as described by Huber et al. (17). Growth was determined by direct cell counting with a Thoma chamber (depth, 0.02 mm).

For platinum shadowing and for embedding, cells were fixed directly in the culture medium with 2% formaldehyde and 2.5% glutardialdehyde. Cells were applied onto a carbon-coated grid, washed once with double-distilled water, and shadowed with 1-nm Pt-C at an angle of 15°. For thin sectioning, cells were harvested by centrifugation, dehydrated, and infiltrated in successive 10-min incubations with 50% ethanol, 70% ethanol, 95% ethanol plus LR White (1 plus 2), and finally pure LR White (2×). The resin was polymerized at 4°C by addition of LR White accelerator. Sections were contrasted with uranyl acetate and lead citrate. Cells for freeze fracturing were frozen in liquid nitrogen in a thin sandwich between two copper plates, cleaved and freeze-etched at −98°C for 3 min in a Cressington CFE 50 freeze-etching apparatus, and shadowed unidirectionally with 1-nm Pt-C (angle, 45°) and 10-nm carbon (90°). Replicas were cleaned on 70% H_2SO_4 overnight. The protein surface layer was isolated by extraction of whole cells with 2% sodium dodecyl sulfate at 60°C and differential centrifugation. Electron micrographs were recorded with a Philips CM12 equipped with a Gatan TV camera, linked to an on-line image processing system (Tietz, Gauting, Germany).

Analysis of metabolic products. Nitrate, nitrite, nitric oxide (NO), NH_3, and H_2S were analyzed as described previously (16). N_2 and nitrous oxide (N_2O) were analyzed qualitatively on a Hewlett-Packard 5890 gas chromatograph with a Porapack QS column (100/120 mesh) with argon as the carrier gas (injector temperature, 70°C; oven temperature, 60°C; detector temperature, 220°C).

Determination of catalase. About 100 μl of a 3% (vol/vol) H_2O_2 solution was dropped onto single colonies. The development of gas bubbles indicated the presence of catalase.

DNA isolation and base composition. DNA was prepared as described by Lauerer et al. (20). The G+C content was determined by melting point analysis (22) and by direct analysis of the nucleosides. All enzymes for nucleoside preparations were purchased from Boehringer, Mannheim, Germany. A total of 12 μl of DNase-free RNase (10 mg/ml) was added to 25 μg of DNA and incubated at 37°C for 30 min. The DNA was precipitated with ice-cold ethanol and centrifuged for 15 min (Eppendorf centrifuge; 4°C), and the precipitate was solubilized in 25 μl of double-distilled H_2O. After boiling for 2 min and cooling on ice, 50 μl of sodium acetate buffer (pH 5.3), 5 μl of 20 mmol of zinc sulfate, and 3 μl of nuclease P1 (340 U/ml) were added. After 2 h of incubation at 37°C, 5 μl of 100 mmol of glycine buffer (pH 10.4) and 5 μl of desaminase activity-free alkaline phosphatase (200 U/ml) were added and the sample was further incubated for 1 h at 37°C. The nucleosides were separated at 35°C on a Spherisorb 5-μm ODS-2 reverse-phase column (25 by 4.6 mm) by high-performance liquid chromatography and detected at 254 nm. The running buffer contained 50 mmol of KH_2PO_4, 10 mmol of tetrabutylammonium hydroxide, and 3% methanol. As references, calf thymus DNA (42 mol% G+C) and lambda DNA (49.8 mol% G+C) were used.

Lipid analysis. Lipids were extracted from lyophilized cells of isolate IM2 as described by De Rosa et al. (11). Total lipid extracts were hydrolyzed with methanol-HCl (90:10, vol/vol) to cleave the polar head groups. The chloroform-soluble fraction of the methanolysis mixture was purified as described previously (10). Thin-layer chromatography was performed on 0.25-mm layers of silica gel 60 F 254 (Merck). Solvents included $CHCl_3$-MeOH-H_2O (65:25:4, by volume) for complex lipids and *n*-hexane–ethyl acetate (75:25 and 80:20, vol/vol) for glycerol dialkyl glycerol tetraethers and glycerol dialkyl glycerol diethers, respectively. For glycerol dialkyl nonitol tetraethers, a solvent of $CHCl_3$-MeOH (90:10, by volume) was used. All compounds were detected by spraying with 0.1% $Ce(SO_4)_2$ in 2 N H_2SO_4 and heating (180°C; 5 min). Quinones were analyzed as described previously (32).

16S rRNA analysis. A detailed phylogenetic analysis of the 16S rRNA sequences of isolate IM2 and *P. islandicum* is described elsewhere (7). The sequences were aligned to a collection of archaeal 16S rRNA sequences (Ribosomal Database Project [RDP], University of Illinois, Urbana). Pairwise evolutionary distances were computed from percent similarities with the correction of Jukes and Cantor (19) as modified by Olsen (33). Only positions in which a known nucleotide is present in all species were used in the calculation.

Nucleotide sequence accession numbers. The sequences used for calculation of evolutionary distances have been submitted to GenBank. Accession numbers are as follows: *Pyrobaculum aerophilum*, L07510; *P. islandicum*, L07511; *Pyrodictium occultum*, M21087; *Sulfolobus solfataricus*, X03235; *Thermococcus celer*, M21529; *Thermofilum pendens*, X14835; *Thermoproteus tenax*, M35966.

RESULTS

Sampling and isolation. Five samples of sand-water mixtures were taken from several different hot, strongly gassed marine water holes at the tidal zone of Maronti Beach in Ischia, Italy. The original temperatures and pH values were from 97 to 102°C and 5.5 to 6.5, respectively. The sample material was transferred into 28-ml serum tubes, which were then tightly stoppered. In three samples, oxygen was reduced by adding sodium sulfide to a final concentration of 0.05% (wt/vol).

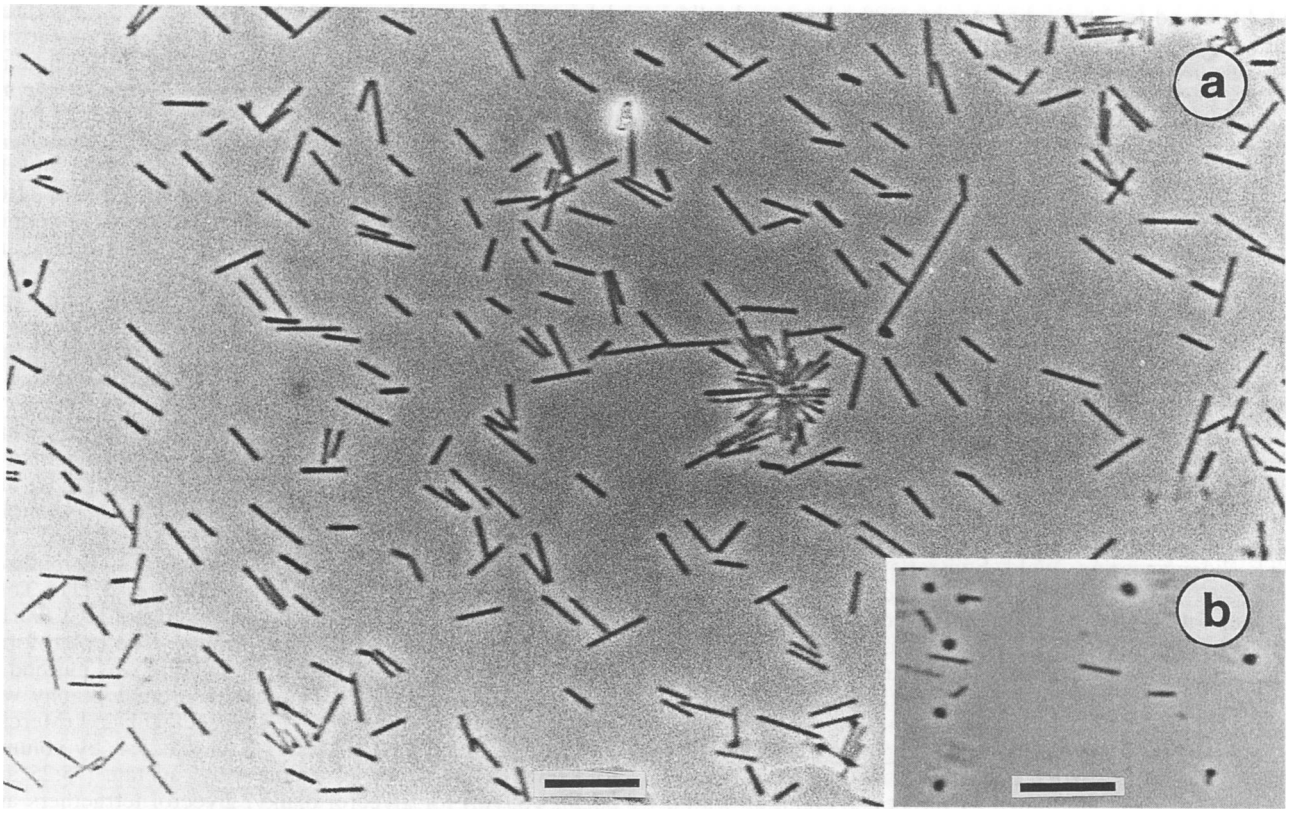

FIG. 1. Phase-contrast micrographs of cells of isolate IM2. (a) Cell population in the mid-log phase. (b) Cells grown in the presence of 2 mM KNO_2. Bars, 10 μm.

In the laboratory, strictly anaerobic marine medium (gas phase, N_2-CO_2; Bacto Difco 2216) was inoculated with 1 ml of the original samples and incubated at 100°C. After 2 weeks, one enrichment attempt which had been inoculated with an anaerobic sample (IM2) contained about 10^6 rod-shaped cells per ml. The redox indicator resazurin had changed from colorless to pink and nitrite was detected as a product. When the enrichment culture was transferred into strictly anaerobic BSY medium (gas phase, N_2-CO_2) supplemented with 0.1% KNO_3, growth with final cell concentrations of about 10^8/ml was obtained at 100°C.

For cloning, the enrichment culture was plated anaerobically onto the same medium, solidified with 6 g of Gelrite per liter. After 2 weeks of incubation at 85°C (gas phase, N_2-CO_2), round greyish-yellow colonies about 1 mm in diameter had developed. One of these colonies served as inoculum for a liquid culture, which was plated again. From a single colony from that plate, a liquid culture was grown, which served as the inoculum for all further experiments (isolate IM2).

Morphology. Cells of isolate IM2 were cylinder-shaped rods with a width of 0.6 μm and an average length of about 5 μm. Most cells were 3 to 8 μm long (Fig. 1a). Rarely, cells with up to a 20-μm length appeared (Fig. 1a). The cells were motile, and the electron microscope showed monopolar flagellation with a bundle of up to eight flagella (diameter, about 10 nm; Fig. 2). During growth, the rods were often arranged in V-shaped aggregates at various angles (Fig. 1a), and X- and raft-shaped aggregates also occurred. At the end of the exponential-growth phase, about 10% of the rods formed spherical bodies, possibly similar to the "golf clubs" known from *Thermoproteus* (38) and *Pyrobaculum* (14) spp. However, in contrast to *Thermoproteus* spp. in the stationary-growth phase, the terminal bodies tended to enlarge with a simultaneous shortening of the rods. During further incubation, several rods converted completely into spheres with a diameter of approximately 1 μm (Fig. 1b). Cultures grown above pH 8.0 or in the presence of high nitrite concentrations (>2 mmol) contained up to 50% coccoid cells.

In ultrathin sections, cells exhibited a cell wall consisting of a protein surface layer (S-layer) situated upon the cytoplasmic membrane (Fig. 3a). The surface layer was composed of a 5-nm outer layer and a 25-nm inner elongated domain, a spacer, anchoring the surface layer in the cytoplasmic membrane (Fig. 3b). The isolated S-layer showed p6 symmetry and a center-to-center distance of 30 nm (Fig. 4). Its basic architecture in projection, as seen in the correlation average (Fig. 4, bottom), was congruent with the surface layers of the related species *P. islandicum*, *P. organotrophum*, and *T. tenax* (3). Accordingly, after freeze-etching, a distinct regular pattern with p6 symmetry and 30-nm periodicity was visible on the surface of the cells (Fig. 3c). In these preparations, no fracture faces through the cytoplasm were visible, consistent with a membrane rich in tetraether lipids (see "Lipid composition" below) (4).

Metabolism. Isolate IM2 grew under strictly anaerobic conditions with nitrate as the electron acceptor. Maximal cell densities were obtained with complex organics such as yeast extract, meat extract, tryptone, or peptone as substrates (Table 1; Fig. 5). Propionate and acetate served as

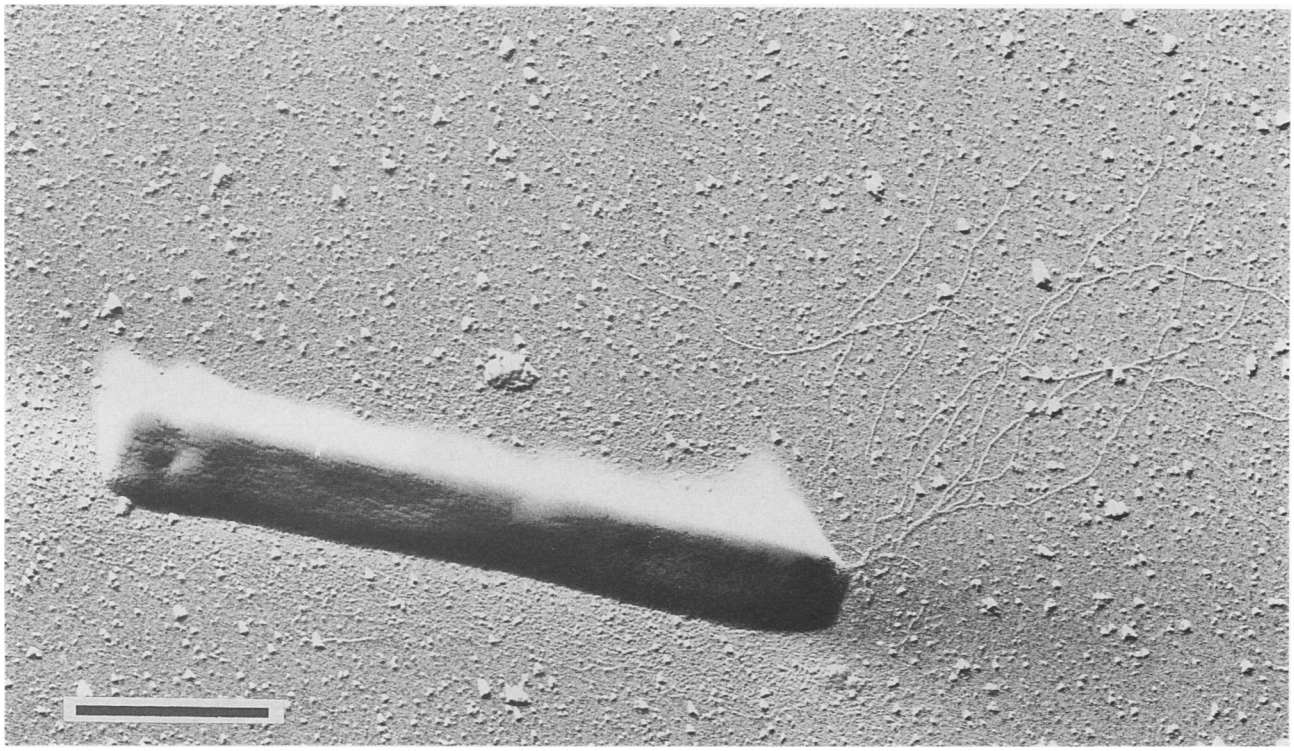

FIG. 2. Electron micrograph of a single cell of isolate IM2, air dried and platinum shadowed. Bar, 1 μm.

single substrates yielding lower final cell densities. In the absence of organic material, isolate IM2 grew chemolithoautotrophically with molecular hydrogen or thiosulfate (Table 1). During growth by nitrate respiration, nitrite was accumulated in the medium (not shown). In addition, traces of nitric oxide (NO) were produced (0.5 ppm). No ammonia was formed. With nitrite as a single electron acceptor in pressurized serum bottles, no growth was obtained. However, within a continuously gassed 1-liter fermentor (N_2-CO_2; 30 ml/min) isolate IM2 grew by nitrite reduction in the presence of organic substrates (Table 1). The products of nitrite reduction were N_2, traces of N_2O, and NO (0.75 ppm). When a mixture of nitrate and nitrite was supplied as the electron acceptors, nitrite was used only after the total consumption of nitrate (Table 2). Nitrous oxide did not serve as an alternative electron acceptor, but it did not inhibit growth by nitrate reduction (up to 50% volume in the gas phase tested). When granules of sulfur were placed onto plates with a lawn of growing cells, a clear inhibition zone (5-mm diameter) became visible after further incubation (not shown). Furthermore, when sulfur (0.5%, wt/vol) was added into exponentially growing liquid cultures cells became granulated about 3 h later and finally lysed. No H_2S was formed during this time. No growth occurred in aerobically prepared medium with and without nitrate. However, the organisms grew in nonreduced medium in the presence of 0.3% oxygen (by volume) in the gas phase (N_2-CO_2), without nitrate reduction. Without nitrate, growth occurred only in the presence of oxygen, indicating that isolate IM2 grew by aerobic respiration. The optimal oxygen concentrations for aerobic growth during lithoautotrophic and organotrophic nutrition were around 0.6 and 1%, respectively (at 300 kPa of N_2-CO_2). The upper limit of growth was between 3 and 5% oxygen (by volume). Without overpressure, vigorous growth was obtained by aerating a 700-ml culture with 5 to 50 ml of air per min. The substrates for aerobic growth were similar to those used during nitrate respiration (Table 1).

Physiological characterization. Isolate IM2 grew between 75 and 104°C with an optimal doubling time of 180 min at 100°C (Fig. 6). At 75°C, the doubling time was about 5 days (not shown). No growth occurred at 70 and 105°C. The temperature limits of growth were the same under aerobic and anaerobic conditions (not shown).

In a chemostat, operated without overpressure, growth was obtained in a pH range from 5.8 to 9.0 with an optimum around 7.0 (Fig. 7). However, in pressurized serum bottles the upper pH limit for growth was 8.0. When the pH of a culture grown at pH 7.0 was adjusted to pH 5.5, rapid cell lysis occurred, as indicated by the appearance of granulated cells in the light microscope and by the vanishing turbidity of the culture.

The optimal salt concentration in the medium was found to be approximately 1.5% NaCl (Fig. 8). Cultures grew up to a concentration of 3.6% NaCl in the medium. However, growth was also observed in trace-element mineral medium without additional salt.

Plating studies. Isolate IM2 was plated onto Gelrite plates under aerobic conditions and was incubated at 92°C. After 4 days, colonies with a diameter of about 1 mm had developed. The plating efficiency approached 100%.

Catalase activity. Single colonies of isolate IM2, grown under aerobic plating conditions, exhibited strong gas production when covered with 3% H_2O_2 solution, indicating the presence of catalase activity. Anaerobically grown colonies showed very poor gas production under these conditions.

Lipid composition. The thin-layer chromatogram of the

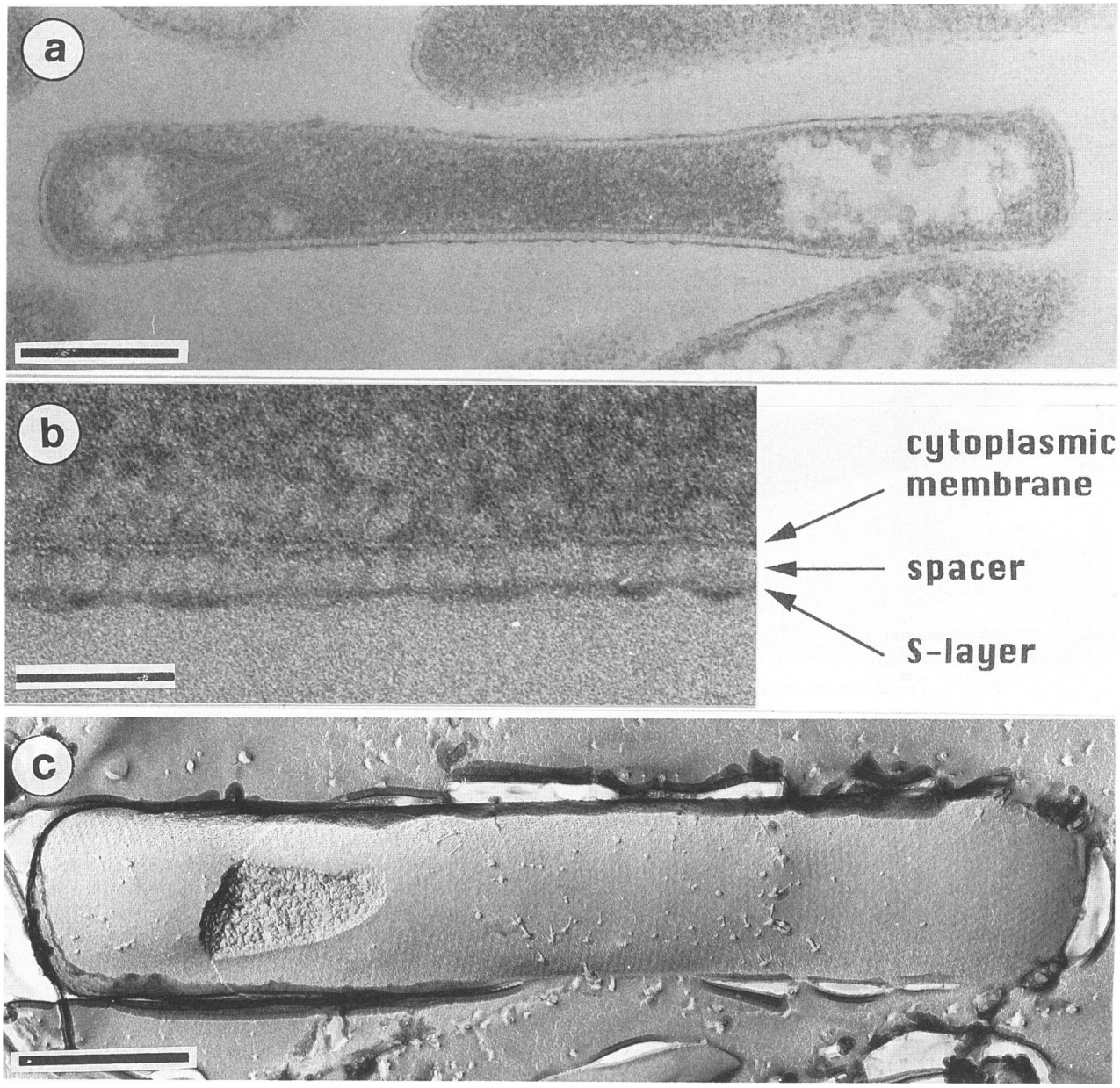

FIG. 3. Ultrathin section (a), enlarged view of section (b), and freeze-etched cell (c) of isolate IM2. Bars, 0.5 μm (a and c) and 0.1 μm (b).

complex lipids of isolate IM2 was very similar to those of *Pyrobaculum* and *Thermoproteus* spp. It showed phosphoglycolipids type I and type II at R_f 0.03 and 0.14. Additional minor unidentified spots were visible at R_f 0.20 and R_f 0.23. The core lipids consisted mainly of acyclic and cyclic glycerol diphytanyl glycerol tetraethers (GDGT) with one to four pentacyclic rings (GDGT, GDGT 0 + 1, 1 + 1, 1 + 2, 2 + 2; R_f 0.37 to 0.53). Rings were found in both aerobically and anaerobically grown cells. Aerobically grown cells exhibited a higher degree of cyclization in their GDGT. In addition, traces of glycerol diphytanyl glycerol diethers were visible under aerobic and anaerobic growth conditions. Glycerol diphytanyl nonitol tetraether, a characteristic lipid structure in members of the *Sulfolobales*, was absent, as were sulfolobusquinone and caldariellaquinone.

DNA base composition. Total DNA of isolate IM2 had a G+C content of 52 mol% as calculated by direct analysis of the mononucleosides as well as by melting point analysis.

Phylogenetic analysis. By comparisons of 16S rRNA sequences, isolate IM2 showed a close relationship to *P. islandicum* as indicated by an estimated exchange of 1.6 per 100 nucleotides (Table 3) (7).

DISCUSSION

The novel marine isolate IM2 represents the first facultative aerobic thermoneutrophilic archaeum (27, 35). It resembles members of *Thermoproteus* and *Pyrobaculum* within the strictly anaerobic sulfur-respiring *Thermoproteales* by its regular rod shape, the formation of terminal spheres (golf

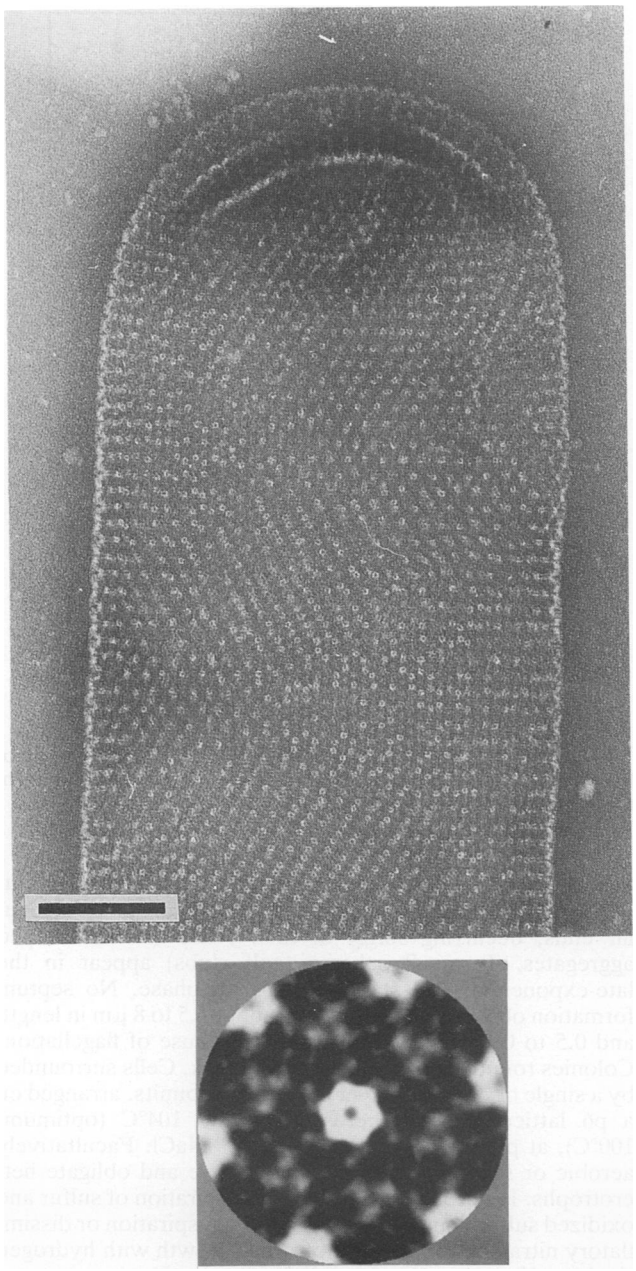

FIG. 4. Electron micrograph of a surface layer fragment from isolate IM2, negatively stained with uranyl acetate. Bar, 0.2 μm. Below, correlation average.

clubs), and its S-layer envelope (3, 15, 23, 38). However, the new isolate was unable to grow by sulfur respiration, and growth was even inhibited by elemental sulfur. It grew by dissimilatory nitrate reduction with H_2, thiosulfate, and organic compounds as substrates. In the presence of complex organics, growth by nitrite reduction was also observed. So far, within the *Archaea*, nitrate reduction has only been found within the strictly heterotrophic extreme halophiles (40). Similar to almost all of the bacterial nitrate reducers, isolate IM2 is a facultative aerobe. In contrast, isolate IM2 grew only at extremely low oxygen tensions. Catalase was formed under microaerobic conditions, and the same substrates as those during nitrate respiration were used. By its 16S rRNA sequence, the new isolate was closely related to *P. islandicum*, with an evolutionary distance of only 1.6%. Therefore, and in line with its morphological features, isolate IM2 represents a new species of *Pyrobaculum* which we name *P. aerophilum* ("the air-loving firestick"). In contrast to their close relationship, the type strains of *P. islandicum*, *P. organotrophum*, *T. tenax*, and *T. neutrophilus* were unable to grow by oxygen and nitrate reduction (data not shown). In addition, they were not capable of growing above 0.8% NaCl and are therefore adapted to low-ionic-strength terrestrial hot environments (14, 15).

TABLE 1. Substrate utilization of isolate IM2 with different electron acceptors in BS medium

Substrate (0.05% final concn)	Electron acceptor[a]		
	KNO_3	KNO_2	O_2[b]
None	−	−	−
Yeast extract	+++	+++	+++
Meat extract	+++	++	+++
Tryptone	+++	++	+++
Peptone	+++	++	+++
Gelatin	++	ND	+
Casein	+	ND	−
Casamino Acids	++	ND	+
Starch	−	ND	−
Sucrose	−	−	−
Glucose	−	−	−
Lactose	−	−	−
Ribose	−	−	−
Pyruvate	−	−	−
Propionate	+	+	+
Acetate	+	+	+
Sodium thiosulfate	+	ND	++
Sulfite	−	ND	−
Sulfur	−	−	−
Sulfur-yeast extract	−	−	−
Hydrogen	+	ND	+

[a] Final cell concentration determined by direct microscopic counting. +++, 10^8 or more cells per ml; ++, 5×10^7 to 9×10^7 cells per ml; +, 1×10^7 to 4×10^7 cells per ml; −, no growth; ND, not determined.

[b] Oxygen concentration with organic and inorganic substrates: 1 and 0.6% by volume, respectively.

The ecological significance of the ability to reduce nitrate is so far unknown since in the laboratory (a) oxygen concentrations as low as 0.3% were sufficient to suppress nitrate reduction of *P. aerophilum* and (b) nitrate may be unstable under reducing conditions in the anaerobic environment (5). Possibly, *P. aerophilum* thrives mainly within hydrothermal systems accessible to oxygen. Because of the low solubility of O_2 at high temperatures, microaerobic environments may exist in boiling marine water holes at the beach, where the isolate had been obtained. On the other hand, even deep hydrothermal systems mixing with oxygen-rich seawater (e.g., in the walls of Black Smokers) can be considered as possible biotopes. Within its environment, *P. aerophilum* is a primary producer of organic matter, most likely giving rise to a food web. In addition, it was able to grow on organic matter and, therefore, may be seen as an opportunistic heterotroph as well.

The phylogenetic position of the new isolate within the *Thermoproteales* poses questions about the history of evolution of nitrate and oxygen respiration on Earth. Within the universal phylogenetic tree, the *Thermoproteales* represent a deep and short lineage, indicating slow evolution (27, 35).

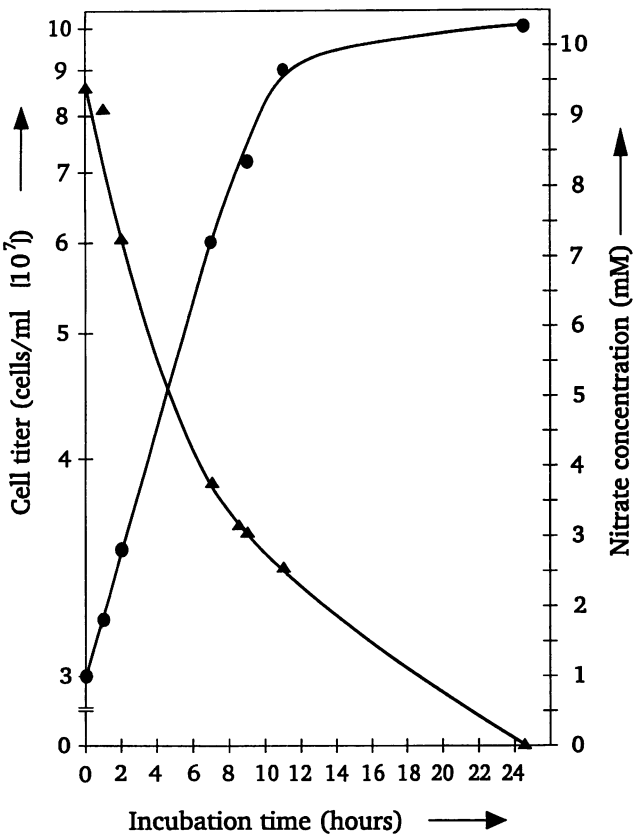

FIG. 5. Nitrate consumption during growth of isolate IM2 in a 700-ml glass fermentor in BSY medium, gassed with 30 ml of N_2-CO_2 per min (97°C). ●, Growth curve; ▲, nitrate consumption.

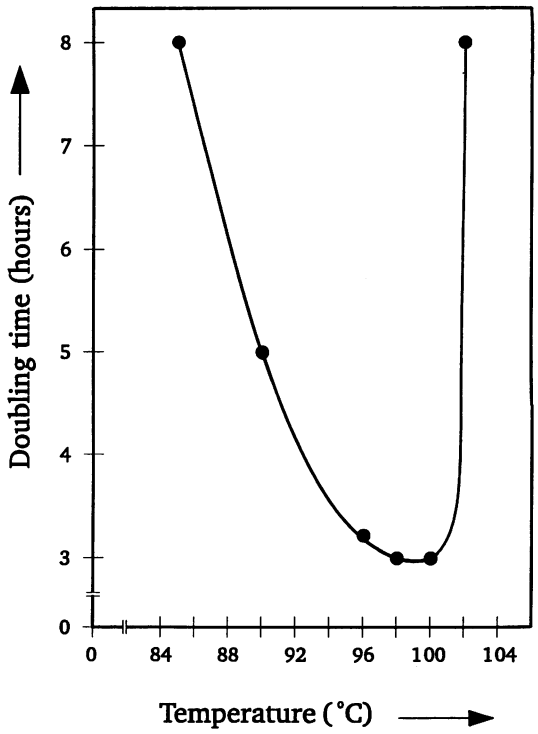

FIG. 6. Optimal growth temperature of isolate IM2 during aerobic growth in BSY medium. Doubling times were calculated from the slopes of the growth curves (not shown).

Therefore, they may still be rather primitive and similar to their archaean ancestors. Interestingly, *A. pyrophilus*, which represents the deepest phylogenetic lineage within the *Bacteria* domain, is a nitrate reducer and microaerophilic H_2 oxidizer, too (16). Possibly, nitrate and traces of oxygen were available on the primitive archaean Earth (21, 31). Therefore, both metabolic types may have evolved well before the transition of Earth to a stable aerobic hydrosphere and atmosphere had occurred. The molecular investigation of nitrate and O_2 respiration of *P. aerophilum* may shed further light on the evolution of these possibly very ancient types of metabolism.

Emended description of the genus *Pyrobaculum*. *Pyrobaculum* Huber, Kristjansson, and Stetter (1986). Emended Huber, Völkl, and Stetter. Cells are rods with almost rectangular ends, occurring singly or in V-, X-, and raft-shaped aggregates. Terminal spheres (golf clubs) appear in the late-exponential and stationary-growth phase. No septum formation observed. Cells usually about 1.5 to 8 μm in length and 0.5 to 0.6 μm in width. Motile because of flagellation. Colonies round and grey to greenish black. Cells surrounded by a single or double S-layer of protein subunits, arranged on a p6 lattice. Growth between 74 and 104°C (optimum, 100°C), at pH 5 to 9, and at 0 to 3.6% NaCl. Facultatively aerobic or strictly anaerobic. Facultative and obligate heterotrophs. Heterotrophic growth by respiration of sulfur and oxidized sulfur compounds, by aerobic respiration or dissimilatory nitrate reduction. Autotrophic growth with hydrogen or thiosulfate by reduction of elemental sulfur, oxygen, or nitrate. Cells are insensitive to penicillin G, streptomycin, phosphomycin, vancomycin, and chloramphenicol and sensitive to rifampin (most likely unspecific inhibition). Elongation factor G is ADP ribosylated. No murein present. Isoprenyl ether lipids in the cell membrane. 44 to 52 mol% G+C. Members of the genus *Pyrobaculum* occur in neutral to slightly alkaline boiling solfataric waters and shallow marine hydrothermal systems. Type species is *Pyrobaculum islandicum* GEO3, DSM 4184, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany.

TABLE 2. Reduction of nitrite after the total consumption of nitrate during anaerobic growth of IM2 in a continuous gassed fermentor[a]

Time of growth (h)	Cells/ml, 10^7	Concn (mM) of:	
		Nitrate	Nitrite
0	5.0	0.05	0.79
1.5	6.2	0.0	0.82
4	6.9	0.0	0.54
6	8.8	0.0	0.48
8	9.7	0.0	0.26
22.5	9.8	0.0	0.07

[a] N_2-CO_2; 80:20, vol/vol.

Description of *Pyrobaculum aerophilum*. *Pyrobaculum aerophilum* Völkl, Huber, and Stetter sp. nov. (a.e.ro'phi.lum. Gr. masc. n. *aer*; adj. *philos*, loving; M.L. neut. adj. *aerophilum*, air loving, because of its ability to use oxygen for growth in contrast to all other described species of the genus *Pyrobaculum*). Cells usually between 3 and 8 μm long; cylinder-shaped rods with a width of 0.6 μm. Motile by

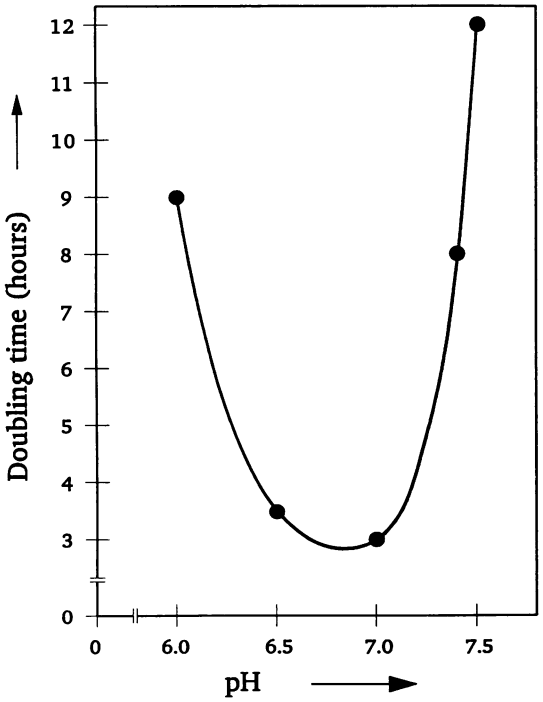

FIG. 7. Influence of pH on aerobic growth of isolate IM2. Doubling times were calculated as for Fig. 6.

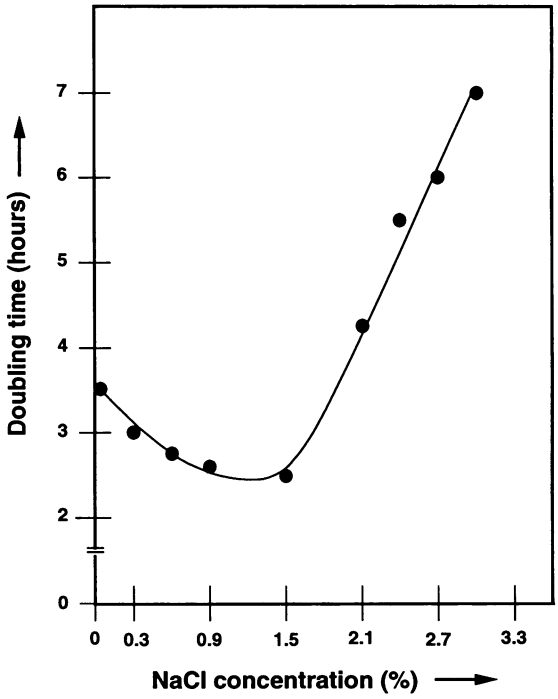

FIG. 8. Effect of NaCl on aerobic growth of isolate IM2. Doubling times were calculated as for Fig. 6.

monopolar flagellation. Cells with terminal spheres occur mainly in the stationary-growth phase, at high nitrite concentrations, or at pH values above 8. Spheres can enlarge, producing coccoid cell forms. Colonies greyish yellow, round with a rough surface. Anaerobically grown, packed cell masses exhibit a deep green color; aerobically grown cells exhibit a brownish yellow color. Growth between 75 and 104°C (optimum, 100°C), at pH 5.8 to 9 (optimum, 7.0), and at 0 to 3.6% NaCl (optimum, 1.5%). Optimal doubling time, 180 min. Heterotrophic growth on yeast extract, meat extract, tryptone, peptone, gelatin, casein, Casamino Acids, propionate, and acetate. Autotrophic growth by oxidation of hydrogen or thiosulfate. Oxygen, nitrate, and nitrite are possible electron acceptors. Nitrite and dinitrogen are products of dissimilatory nitrate reduction (denitrification). Growth inhibition by elemental sulfur. Aerobically grown cells exhibit catalase-positive reaction. Cell wall composed of a protein surface layer, exhibiting p6 symmetry and a center-to-center distance of approximately 30 nm between neighboring protein complexes. Core lipids mainly glycerol diphytanyl glycerol tetraethers with varying degrees of cyclization. DNA base composition, 52 mol% G+C. By 16S rRNA sequence comparisons, 1.6% evolutionary distance to *P. islandicum*. Type strain: *Pyrobaculum aerophilum* IM2, DSM 7523, Braunschweig, Germany (isolated at Maronti Beach, Ischia, Italy).

TABLE 3. Evolutionary distances (estimated changes per 100 nucleotides) among various archaea

Organism	*Pyrobaculum aerophilum*	*Pyrobaculum islandicum*	*Thermoproteus tenax*	*Thermofilum pendens*	*Pyrodictium occultum*	*Sulfolobus solfataricus*
Pyrobaculum aerophilum						
Pyrobaculum islandicum	1.6					
Thermoproteus tenax	4.0	4.6				
Thermofilum pendens	12.9	12.3	13.0			
Pyrodictium occultum	13.4	13.0	12.3	12.4		
Sulfolobus solfataricus	17.4	16.9	17.6	17.4	12.7	
Thermococcus celer	21.0	19.9	21.2	19.1	18.7	25.4

ACKNOWLEDGMENTS

We greatly appreciate the help and technical assistance of H. Segerer, P. Hummel, H. Faust, Edoardo Pagnotta, and K. Eichinger. Furthermore, we thank P. Markiewicz and H. Jannasch for critical reading of the manuscript.

This work was supported by grants of the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie to K. O. Stetter.

REFERENCES

1. **Balch, W. E., G. E. Fox, L. J. Magrum, C. R. Woese, and R. S. Wolfe.** 1979. Methanogens: reevaluation of a unique biological group. Microbiol. Rev. **43**:260–296.
2. **Balch, W. E., and R. S. Wolfe.** 1976. New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. Appl. Environ. Microbiol. **32**:781–791.
3. **Baumeister, W., and G. Lembcke.** 1992. Structural features of archaebacterial cell envelopes. J. Bioenerg. Biomembr. **24**:567–575.
4. **Beveridge, T., C. G. Choquet, G. B. Patel, and G. D. Sprott.**

1993. Freeze-fracture planes of methanogen membranes correlate with the content of tetraether lipids. J. Bacteriol. **175**:1191–1197.

5. **Blöchl, E., M. Keller, G. Wächtershäuser, and K. O. Stetter.** 1992. Reactions depending on iron sulfide and linking geochemistry with biochemistry. Proc. Natl. Acad. Sci. USA **89**:8117–8120.
6. **Bonch-Osmolovskaya, E. A., M. L. Miroshnichenko, N. A. Kostrikina, N. A. Chernych, and G. A. Zavarzin.** 1990. *Thermoproteus uzoniensis* sp. nov., a new extremely thermophilic archaebacterium from Kamchatka continental hot springs. Arch. Microbiol. **154**:556–559.
7. **Burggraf, S., N. Larsen, C. R. Woese, and K. O. Stetter.** 1993. An intron within the 16S ribosomal RNA gene of the archaeon *Pyrobaculum aerophilum*. Proc. Natl. Acad. Sci. USA **90**:2547–2550.
8. **Burggraf, S., G. J. Olsen, K. O. Stetter, and C. R. Woese.** 1992. A phylogenetic analysis of *Aquifex pyrophilus*. Syst. Appl. Microbiol. **15**:352–356.
9. **Darland, G., T. D. Brock, W. Samsonoff, and S. F. Conti.** 1970. A thermophilic acidophilic *Mycoplasma* isolated from a coal refuse pile. Science **170**:1416–1418.
10. **De Rosa, M., A. Gambacorta, R. Huber, V. Lanzotti, B. Nicolaus, K. O. Stetter, and A. Trincone.** 1989. Lipid structures in *Thermotoga maritima*, p. 167–173. *In* M. S. Da Costa, J. C. Duarte, and R. A. D. Williams (ed.), Microbiology of extreme environments and its potential for biotechnology. Elsevier Applied Science, London.
11. **De Rosa, M., A. Gambacorta, B. Nicolaus, B. Chappe, and P. Albrecht.** 1983. Isoprenoid ethers: backbone of complex lipids of the archaebacterium *Sulfolobus solfataricus*. Biochim. Biophys. Acta **753**:249–256.
12. **Fischer, F., W. Zillig, K. O. Stetter, and G. Schreiber.** 1983. Chemolithoautotrophic metabolism of anaerobic extremely thermophilic archaebacteria. Nature (London) **301**:511–513.
13. **Huber, G., E. Drobner, H. Huber, and K. O. Stetter.** 1992. Growth by aerobic oxidation of molecular hydrogen in *archaea*—a metabolic property so far unknown for this domain. Syst. Appl. Microbiol. **15**:502–504.
14. **Huber, R., J. K. Kristjansson, and K. O. Stetter.** 1987. *Pyrobaculum* gen. nov., a new genus of neutrophilic, rod-shaped archaebacteria from continental solfataras growing optimally at 100°C. Arch. Microbiol. **149**:95–101.
15. **Huber, R., and K. O. Stetter.** 1992. The order *Thermoproteales*, p. 677–683. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), The prokaryotes, 2nd ed., vol. I. Springer-Verlag, New York.
16. **Huber, R., T. Wilharm, D. Huber, A. Trincone, S. Burggraf, H. König, R. Rachel, I. Rockinger, H. Fricke, and K. O. Stetter.** 1992. *Aquifex pyrophilus* gen. nov. sp. nov., represents a novel group of marine hyperthermophilic hydrogen-oxidizing bacteria. Syst. Appl. Microbiol. **15**:340–351.
17. **Huber, R., C. R. Woese, T. A. Langworthy, H. Fricke, and K. O. Stetter.** 1989. *Thermosipho africanus* gen. nov. represents a new genus of thermophilic eubacteria within the "*Thermotogales*." Syst. Appl. Microbiol. **12**:32–37.
18. **Huber, R., C. R. Woese, T. A. Langworthy, J. K. Kristjansson, and K. O. Stetter.** 1990. *Fervidobacterium islandicum* sp. nov., a new extremely thermophilic eubacterium belonging to the "*Thermotogales*." Arch. Microbiol. **154**:105–111.
19. **Jukes, T. H., and C. R. Cantor.** 1969. Evolution of protein molecules, p. 21–132. *In* H. N. Munro (ed.), Mammalian protein metabolism. Academic Press, New York.
20. **Lauerer, G., J. K. Kristjansson, T. A. Langworthy, H. König, and K. O. Stetter.** 1986. *Methanothermus sociabilis* sp. nov., a second species within the *Methanothermaceae* growing at 97°C. Syst. Appl. Microbiol. **8**:100–105.
21. **Mancinelli, R. L., and L. I. Hochstein.** 1986. The occurrence of denitrification in extremely halophilic bacteria. FEMS Microbiol. Lett. **35**:55–58.
22. **Marmur, J., and P. Doty.** 1962. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. J. Mol. Biol. **5**:109–118.
23. **Phipps, B. M., R. Huber, and W. Baumeister.** 1991. The cell envelope of the hyperthermophilic archaebacterium *Pyrobaculum organotrophum* consists of two regularly arrayed protein layers: three-dimensional structure of the outer layer. Mol. Microbiol. **5**(2):253–265.
24. **Segerer, A. H., and K. O. Stetter.** 1992. The genus *Thermoplasma*, p. 712–718. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), The prokaryotes, 2nd ed., vol. I. Springer-Verlag, New York.
25. **Segerer, A. H., and K. O. Stetter.** 1992. The order *Sulfolobales*, p. 684–701. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), The prokaryotes, 2nd ed., vol. I. Springer-Verlag, New York.
26. **Stetter, K. O.** 1989. Order III. *Sulfolobales* ord. nov., p. 2250. *In* J. T. Staley, M. P. Bryant, N. Pfennig, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 3. Williams & Wilkins, Baltimore.
27. **Stetter, K. O.** 1993. Life at the upper temperature border, p. 195–219. *In* J. & K. Tran Than Van, J. C. Mounolou, J. Schneider, and C. McKay (ed.), Colloque Interdisciplinaire du Comité National de la Recherche Scientifique, Frontiers of Life, C55. Editions Fronières, Gif-sur-Yvette, France.
28. **Stetter, K. O., G. Fiala, G. Huber, R. Huber, and A. Segerer.** 1990. Hyperthermophilic microorganisms. FEMS Microbiol. Rev. **75**:117–124.
29. **Tindall, B. J.** 1992. The family *Halobacteriaceae*, p. 768–808. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), The prokaryotes, 2nd ed., vol. I. Springer-Verlag, New York.
30. **Tomlinson, G. A., L. L. Jahnke, and L. I. Hochstein.** 1986. *Halobacterium denitrificans* sp. nov., an extremely halophilic denitrifying bacterium. Int. J. Syst. Bacteriol. **36**:66–70.
31. **Towe, K. M.** 1990. Aerobic respiration in the archaean? Nature (London) **348**:54–56.
32. **Trincone, A., B. Nicolaus, G. Palmieri, M. De Rosa, R. Huber, G. Huber, K. O. Stetter, and A. Gambacorta.** 1992. Distribution of complex and core lipids within new hyperthermophilic members of the *archaea* domain. Syst. Appl. Microbiol. **15**:11–17.
33. **Weisburg, W. G., J. G. Tully, D. L. Rose, J. P. Petzel, H. Oyaizu, D. Yang, L. Mandelco, J. Sechrest, T. G. Lawrence, J. Van Etten, J. Maniloff, and C. R. Woese.** 1989. A phylogenetic analysis of the *Mycoplasmas*: basis for their classification. J. Bacteriol. **171**:6455–6467.
34. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51**:221–271.
35. **Woese, C. R., L. Achenbach, P. Rouvière, and L. Mandelco.** 1991. Archaeal phylogeny: reexamination of the phylogenetic position of *Archaeoglobus fulgidus* in light of certain composition-induced artefacts. Syst. Appl. Microbiol. **14**:364–371.
36. **Woese, C. R., O. Kandler, and M. L. Wheelis.** 1990. Towards a natural system of organisms: proposal for the domains *Archaea*, *Bacteria* and *Eucarya*. Proc. Natl. Acad. Sci. USA **87**:4576–4579.
37. **Zillig, W., A. Gierl, G. Schreiber, S. Wunderl, D. Janekovic, K. O. Stetter, and H. P. Klenk.** 1983. The archaebacterium *Thermofilum pendens* represents a novel genus of thermophilic, anaerobic sulfur-respiring *Thermoproteales*. Syst. Appl. Microbiol. **4**:79–87.
38. **Zillig, W., K. O. Stetter, W. Schäfer, D. Janekovic, S. Wunderl, I. Holz, and P. Palm.** 1981. *Thermoproteales*: a novel type of extremely thermoacidophilic anaerobic archaebacteria isolated from Islandic solfataras. Zentralbl. Bakteriol. Hyg. Abt. 1 Orig. **C2**:200–227.
39. **ZoBell, C. E.** 1941. Studies on marine bacteria. I. The cultural requirements of heterotrophic aerobes. J. Mar. Res. **4**:42–75.
40. **Zumft, W. G.** 1992. The denitrifying prokaryotes, p. 554–582. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), The prokaryotes, 2nd ed., vol. I. Springer-Verlag, New York.